# THE EQUILIBRIUM OF OXYGEN WITH THE HEMOCYANIN OF *LIMULUS POLYPHEMUS* DETERMINED BY A SPECTROPHOTOMETRIC METHOD

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The respiratory proteins, including hemoglobin, hemocyanin, chlorocrurin and hemerythryn, are unique in combining with and dissociating from oxygen at pressures which fit them for the physiological transportation of this gas. The factors which determine the condition of equilibrium between oxygen and the pigment are of interest not only because of the evident physiological relationship between the characteristics of the oxygen dissociation curves of the blood of various organisms and the pressures of oxygen in the environment, but because of the interesting physico-chemical problem which the phenomena present. The hemocyanins appear to possess certain advantages for the study of these problems. Not only do these proteins exist naturally in solution in the blood so that the complications which arise from dealing with corpuscles are avoided, but they are relatively stable compounds which lend themselves without difficulty to purification and preservation. The hemocyanins of different species appear, in addition, to exhibit very considerable differences in their physical and chemical properties; and consequently, one has the advantage in their study of being able to resort to the comparative method in testing generalizations. Finally, from the technical point of view, the hemocyanins which are essentially colorless when reduced become strongly colored in the oxygenated state and consequently lend themselves to the employment of colorimetric methods for the determination of the degree of oxygenation of the solutions.

The present paper contains an account of a spectrophotometric method for the determination of the degree of oxygenation of hemocyanin solutions. The method is applied to an examination of the equilibrium between oxygen and a purified salt-free preparation of the hemocyanin of the horse-shoe crab, *Limulus polyphemus*, at different hydrogen ion concentrations.

## The Spectrophotometric Method

The color of hemocyanin solutions has been taken as an indication of the degree of oxygenation of the protein and used as the basis for

constructing oxygen dissociation curves by Pantin and Hogben (1925) and Redfield and Hurd (1925). The method has an advantage over the usual methods of gas analysis in that it requires no correction for the oxygen dissolved in the solution—a correction which is relatively large in comparison to the oxygen content in the case of hemocyanin solutions and which is difficult to determine in a satisfactory manner. When submitted to the proper controls, the method has the advantage that it measures oxyhemocyanin directly by the employment of the spectrophotometer to determine the absorption of monochromatic light of suitable wave-length. Measurements can be made with an ease and accuracy not obtained in the available methods of gas analysis when applied to hemocyanin solutions.

The study of the absorption of light by hemocyanin solutions (Redfield, 1930) affords the essential basis for the employment of the spectrophotometric method. It was shown that the absorption spectra may be analyzed into two components. One is that due to the scattering of light by the solution, the other is that attributable to the true absorption by the chromatic group formed when oxygen unites with the hemocyanin molecule to form oxyhemocyanin. In addition, with the blood of certain animals, the presence of other coloring matters must be taken into account. The component due to the scattering of light is variable depending upon the composition of the solution. It may, however, be readily determined by the study of reduced solutions. The component due to true absorption by the chromatic group was found to be a constant, characteristic of the amount of oxyhemocyanin present, and to vary little if at all with changes in the solution. The presence of other pigments in the blood does not offer complications to the spectrophotometric determination of the absorption of light by the chromatic group, provided these pigments do not undergo change in color with oxygenation; and their influence upon the measurements may be largely avoided by selecting for measurement wave-lengths which are little absorbed by these pigments.

In order to confirm the assumption that the color of a hemocyanin solution is an index of the quantity of oxygen combined with the protein (a supposition heretofore entirely unsupported by exact experiment), the degree of oxygenation of the serum of the horse-shoe crab, *Limulus polyphemus,* was determined simultaneously by the colorimetric method and with the Van Slyke blood-gas analyzer, at a series of oxygen pressures insufficient to produce complete saturation. To 90 cc. of fresh serum, 10 cc. of 0.05 NaOH were added. The pH value of this solution was pH 8.3; the oxygen content was 1.4 volumes per cent when equilibrated with air. Specimens of 10 cc. of serum were equilibrated

with air in tonometers evacuated to varying degrees, after the method described by Pantin and Hogben (1925). The tonometers consisted of 250 cc. cylindrical vessels provided with a small test tube sealed on at one end. The other end was closed with a rubber stopper provided with a two-way glass stopcock. When equilibration was complete, the hemocyanin was run down into the small test tube and its color compared with a series of standards made up by diluting the original serum as described by Pantin and Hogben. The tonometers were connected with a reservoir of hydrogen and this gas was allowed to flow in until the pressure was raised to that of the atmosphere. The sample was now withdrawn from the tonometer into a pipette and transferred to a Van Slyke blood-gas analyzer, with which its oxygen content was measured. In estimating the oxygen dissolved in the samples, the solubility coefficient was taken to be 0.0235 (Redfield, Coolidge and Montgomery, 1928), a value closely checked by direct measurements on this specimen of serum.

TABLE I

*Comparison of Colorimetric and Gasometric Determination of Degree of Saturation of Limulus Serum with Oxygen*

| $O_2$ Pressure | $O_2$ Content | $O_2$ Dissolved | $O_2$ Combined | Saturation | Color |
|---|---|---|---|---|---|
| *mm. Hg* | *vol. per cent* | *vol. per cent* | *vol. per cent* | *per cent* | *per cent* |
| 3.1 | 0.082 | 0.010 | 0.072 | 7.8 | 10 |
| 4.5 | 0.152 | 0.014 | 0.138 | 15.0 | 20 |
| 5.3 | 0.235 | 0.016 | 0.219 | 23.8 | 25 |
| 10.8 | 0.399 | 0.034 | 0.365 | 39.7 | 40 |
| 13.8 | 0.484 | 0.043 | 0.441 | 48.0 | 50 |
| 15.9 | 0.548 | 0.049 | 0.499 | 54.4 | 55 |
| 21.7 | 0.653 | 0.067 | 0.586 | 63.8 | 65 |
| 34.4 | 0.953 | 0.106 | 0.847 | 92.0 | 90 |
| 155.0 | 1.400 | 0.480 | 0.920 | 100.0 | 100 |

The results of this experiment are recorded in Table I. It may be seen that the degree of saturation of the solution as estimated from its color agrees closely with that determined from the direct measurement of the oxygen combined with the hemocyanin. The use of the spectrophotometer would markedly improve the precision of the colorimetric estimations in this experiment. The errors inherent in the gasometric measurements are, however, so large that the significance of the comparison would not be increased by the further refinement of this part of the experiment.

While the foregoing affords practical demonstration of the utility of colorimetric methods for determining the oxygenation of hemocyanin solutions, confidence in the more precise measurements obtained with the spectrophotometer must be based upon the theoretical adequacy of the procedure.

The analysis of the absorption of light by hemocyanin solutions indicated that, for any wave-length of light

$$E_o = E_x + E_r, \tag{1}$$

where $E_o$ is the extinction coefficient of the oxygenated solution, $E_x$ is the extinction coefficient characterizing the absorption of light by the chromatic groups in the oxygenated solution, and $E_r$ is the extinction coefficient of the reduced solution. It was shown that Beer's law applies to hemocyanin solutions in both the oxygenated and reduced condition. One may consequently write:

$$E_o = cK_o,$$
$$E_r = cK_r,$$
$$E_x = cK_x,$$

where $c$ is the concentration of hemocyanin and $K_o$, $K_r$, and $K_x$ are the extinction coefficients at unit concentration. It follows that

$$E_o = cK_x + cK_r. \tag{2}$$

If $E_y$ be the extinction coefficient characteristic of a mixture of oxygenated and reduced hemocyanin in which the concentration of oxygenated hemocyanin is $yc$ and that of reduced hemocyanin is $(1 - y)c$,

$$E_y = y(cK_x + cK_r) + (1 - y)cK_r, \tag{3}$$
$$E_y = ycK_x + cK_r. \tag{4}$$

Substituting $E_r$ for $cK_r$ in equations (2) and (4), dividing and rearranging,

$$y = \frac{E_y - E_r}{E_o - E_r}. \tag{5}$$

This result is obtained without any explicit assumption regarding the cause for the absorption of light measured by $E_r$, and the equation may consequently be applied in determining the degree of saturation of solutions in which other pigments as well as scattering effects are responsible for the value of this term. It may also be derived by means of a slightly different argument for cases such as that exhibited by hemoglobin solutions, in which the prosthetic group absorbs considerable but different quantities of light in the oxygenated and reduced condition.

It is assumed in the foregoing that an incompletely saturated solution of hemocyanin is a mixture of completely reduced and completely oxygenated elements. No account is taken of the possibility that incompletely oxygenated molecules may occur which possess absorption spectra different from that of the completely oxygenated solution.

This possibility cannot be ignored in view of the success which theories of intermediate degrees of oxygenation have met in explaining the characteristics of the oxygen dissociation curves of hemoglobin (Adair, 1925; Ferry and Green, 1929), even though the experiments of Conant and McGrew (1930) failed to demonstrate the existence of such intermediate compounds. The high molecular weights reported for hemocyanins (Svedberg and Chirnoaga, 1928; Svedberg and Heyroth, 1929) definitely indicate that many oxygen molecules may combine with each hemocyanin molecule. If the chromatic group undergoes intermediate degrees of oxygenation, this will affect the foregoing deduction only in so far as the spectrum of the partially oxygenated chromatic group differs from that of the completely oxygenated chromatic group. The spectrum of the chromatic group in a partially saturated solution has consequently been determined and compared with that of the completely oxygenated solution. The result is tabulated in Table II. The ratio of the extinction coefficients of the chromatic group of fully and partially oxygenated solutions is practically the same at all wave lengths, which indicates that the partially saturated solution does not contain intermediate compounds which differ in their spectral characteristics from the fully oxygenated solution.

TABLE II

*Comparison of Spectrum of Fully Oxygenated and Partially Oxygenated Hemocyanin of Limulus.* Concentration, .0258 grams per cc.; length of tube, 3.3 cm.; pH = 7.43; "Salt Free."

| Wave-Length | Equilibrated with 726 mm. $O_2$ | Equilibrated with 2.6 mm. $O_2$ | Equilibrated with $H_2$ | Oxygenated Chromatic Group | Partially Oxygenated Chromatic Group | $y$ |
|---|---|---|---|---|---|---|
| $m\mu$ | $E_o$ | $E_y$ | $E_r$ | $E_o-E_r$ | $E_y-E_r$ | $\frac{E_y-E_r}{E_o-E_r}$ |
| 460 | 0.157 | 0.100 | 0.034 | 0.123 | 0.066 | .536 |
| 480 | 0.136 | 0.086 | 0.034 | 0.102 | 0.052 | .510 |
| 500 | 0.151 | 0.094 | 0.030 | 0.121 | 0.064 | .529 |
| 520 | 0.199 | 0.120 | 0.028 | 0.171 | 0.092 | .538 |
| 540 | 0.253 | 0.150 | 0.026 | 0.227 | 0.124 | .546 |
| 560 | 0.288 | 0.170 | 0.026 | 0.262 | 0.144 | .550 |
| 580 | 0.303 | 0.178 | 0.027 | 0.276 | 0.151 | .547 |
| 600 | 0.302 | 0.172 | 0.025 | 0.277 | 0.147 | .530 |
| 620 | 0.285 | 0.168 | 0.025 | 0.260 | 0.143 | .550 |
| 640 | 0.265 | 0.155 | 0.024 | 0.241 | 0.131 | .544 |
| 660 | 0.244 | 0.143 | 0.028 | 0.216 | 0.115 | .532 |
| 680 | 0.216 | 0.127 | 0.028 | 0.188 | 0.099 | .528 |
| 700 | 0.200 | 0.117 | 0.022 | 0.178 | 0.095 | .530 |

## The Determination of the Oxygen Dissociation Curve

*Measurement of Degree of Oxygenation.*—The extinction coefficients of the solutions were measured with the aid of a König-Martens spectrophotometer. They are given by equations of the type

$$E_o = \frac{2(\log \tan a_o - \log \tan a_1)}{d}, \tag{6}$$

where $a_o$ is the angle of the analyzing Nicol prism when oxyhemocyanin is measured; $a_1$ is the angle when the absorption of light by the solvent is determined; and $d$ is the length of the absorbing column. Similarly, expressing the angular reading characteristic of an incompletely saturated solution as $a_y$ and that of the reduced solution as $a_r$, $E_y$ and $E_r$ are obtained. Substituting in (5) the degree of saturation, $y$ is given by the expression

$$y = \frac{\frac{2}{d}\log \tan a_y - \frac{2}{d}\log \tan a_r}{\frac{2}{d}\log \tan a_o - \frac{2}{d}\log \tan a_r}. \tag{7}$$

Since the corrections for the absorption of light by the solvent cancel out, they need not be measured. In practice the greatest accuracy is obtained at wave-lengths giving the greatest difference between the values of $E_o$ and $E_r$. In the case of hemocyanin solutions, this occurs in the yellow region of the spectrum. This fact is fortunate in that this is the region in which readings can be made with the greatest accuracy. It is also a practical advantage that the scattering of light is small at these wave lengths and variations due to changes in the physical conditions of the solution are consequently minimal. In preparing solutions, the greatest precision is obtained by adjusting the concentration and the length of the absorbing chamber so that the angle $a_o$ is as large as is compatible with precise measurement, that is, about 75°.

*Equilibration with Oxygen.*—In order to equilibrate the solutions with oxygen of known pressure, tonometers such as those illustrated in Fig. 1 were employed. These consist of cylindrical bottles of Pyrex glass, having a capacity of 250 cc., to one end of which is sealed a T-tube having an internal diameter of approximately one centimeter. The ends of the T are ground parallel to one another and are closed with flat glass disks sealed on with DeKhotinsky cement. The mouth of the bottle is closed with a rubber stopper in which a two-way glass stopcock is inserted. About five cc. of the solution to be measured is placed in the tonometer, which is then evacuated and refilled with a gas mixture

containing a convenient proportion of oxygen. In the experiments described in this paper, nitrogen containing 2 to 5 per cent of oxygen has been convenient; for other solutions, air will serve or pure oxygen

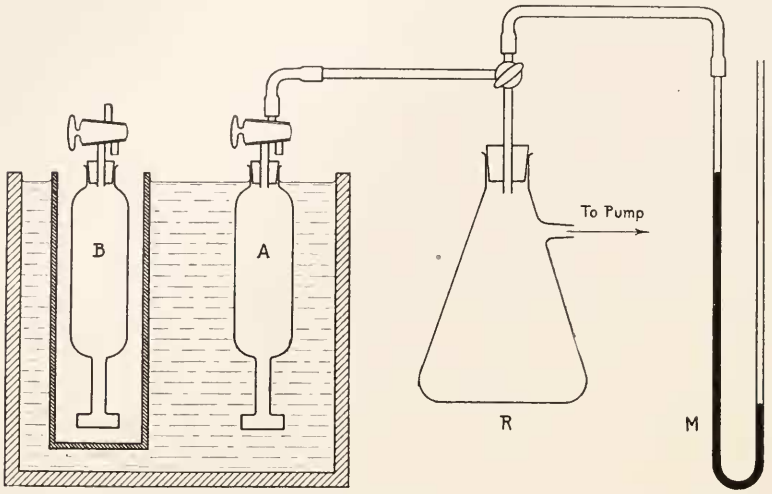


FIG. 1. Arrangement for adjusting pressure of gas in tonometers. *A*, tonometer in position for measuring pressure after equilibration. *B*, tonometer in thermostat during settling of sediment. *M*, mercury manometer. *R*, reservoir which serves to slow rate of evacuation of tonometers.

may be necessary. The tonometer is now evacuated to some definite pressure and the solution equilibrated with the gas at this pressure, rotating the tonometer in a horizontal position for fifteen minutes in a water bath at constant temperature. The tonometer is next returned to a vertical position and evacuated and filled again with the gas mixture and pumped out to the same reduced pressure. The solution is further equilibrated for twenty-five minutes and then, without removal from the water bath, is turned into the vertical position and connected with the manometer after setting the pressure in the system to that expected to obtain in the tonometer. The passage connecting the tonometer and manometer with the pump and reservoir is now closed. The stopcock leading into the tonometer is opened, and the pressure obtaining in the tonometer is carefully measured and recorded. The stopcock of the tonometer is closed again and disconnected from the pump. The tonometer is carefully dried and placed in an air-chamber inserted into the water bath where it is kept for a period of approximately one hour in order that the small particles of denatured protein which almost invariably form during the process of evacuation and equilibration may

settle out. Following this, the absorption of light by the hemocyanin solution is measured by placing the tonometer with the T-tube in the path of one of the beams of the spectrophotometer. If the solutions are not clear at the time when the measurements are made, the results should be rejected.

The partial pressure of oxygen in the tonometer, $p$, is given by the expression

$$p = (B - P - aq)\, f,$$

where $B$ is the barometric pressure, $P$ is the pressure in the tonometer recorded at the end of equilibration, $aq$ is the tension of aqueous vapor at the temperature of the water bath, and $f$ is the fraction of oxygen in the gas mixture.

To obtain complete oxygenation, the tonometer is simply evacuated and filled with pure oxygen gas prior to equilibration. To obtain complete reduction is difficult under those circumstances in which the affinity of hemocyanin for oxygen is great. We have not found the employment of chemical reducing agents satisfactory, as certain of these tend to influence the color of the solution and others must be employed in such concentrations that they may affect the scattering of light on which the absorption by the reduced solution depends. The most satisfactory procedure is to employ hydrogen to wash out the tonometer after the oxygen is freed from the hemocyanin under low pressure. The solutions are accordingly evacuated, equilibrated for twenty minutes, filled with hydrogen, re-evacuated, again equilibrated, allowed to settle, and then measured. Further repetition of the process does not lead to lower readings, although it is doubtful whether, under certain circumstances, this process removes the last traces of oxyhemocyanin. The reason for this is that, in the process of evacuation and equilibration, small quantities of denatured material are formed which fail to settle out completely when the solutions are allowed to stand. The formation of precipitates of this sort, which goes on more readily in the reduced solutions, constitutes the principal limit to the precision of the method. We have recently constructed tonometers which can be placed in the cups of a large centrifuge and which make it possible to remove these troublesome precipitates. Such tonometers have not been employed in the experiments described in this paper.

*The Preparation of the Hemocyanin Solutions.*—The hemocyanin employed in the present investigation was prepared from material obtained during the summer of 1928. It was preserved in the precipitated state by adding 350 grams of ammonium sulphate to each liter of serum. The material was purified some months later by repeated salting out

followed by dialysis, against dilute sodium hydroxide, as described by Redfield, Coolidge and Shotts (1928). Three preparations were obtained, having the following characteristics: Specimen 18 *A,* dry weight 0.1031 gram per cc., combined base $19.4 \times 10^{-5}$ mols per gram; Specimen 18 *B,* dry weight 0.1255 gram per cc., copper 0.0208 milligram per cc. or 0.168 gram per 100 grams dry substance, combined base $19.1 \times 10^{-5}$ mols per gram; Specimen 18 *C,* dry weight 0.097 gram per cc., copper 0.16 milligram per cc. or 0.165 gram per 100 grams dry weight, combined base $21.6 \times 10^{-5}$ mols per gram. These solutions were preserved with toluene at a low temperature. The day before measurements were to be made, they were further diluted by the addition of distilled water containing amounts of hydrochloric acid or sodium hydroxide appropriate to secure the desired hydrogen ion activity and to reduce the hemocyanin to a concentration favorable for the measurements; that is, to about 2.5 per cent. After standing all night, the solutions were filtered and then employed for the determination of the oxygen dissociation curves. A portion of the solution was also reduced by equilibration with hydrogen and used for the determination of the hydrogen ion concentration by means of the hydrogen electrode.[1]

Measurements were made upon solutions at several hydrogen ion activities between pH 7.4 and pH 10.4, and upon a solution at pH 4.5. At hydrogen ion activities intermediate between pH 4.5 and about pH 6.8, *Limulus* hemocyanin is insoluble in distilled water, and solutions of sufficient clarity cannot be obtained. At reactions more acid than pH 4.5, a colorless modification of *Limulus* hemocyanin is formed (Redfield and Mason, 1928). The characteristics of the oxygen dissociation curve at these hydrogen ion activities will be dealt with in a subsequent paper.[2]

## Data on Oxygen Dissociation

The results of the series of measurements which have been made upon purified solutions of *Limulus* hemocyanin are recorded in Table III. The first column contains a description of the material employed in each case; the second column, the partial pressure of oxygen in the tonometer at the completion of equilibration; the third column, the value of the extinction coefficient of the solution ($2/d$ log tan $a_y$), as measured with the spectrophotometer, employing light of the wave-length

[1] In the case of the two solutions prepared from Specimen 18 *C,* the pH value was somewhat less than that to be expected from the amount of NaOH added, as judged from the titration curve published by Redfield, Humphreys and Ingalls (1929). In the other solutions the agreement is good.

[2] I am indebted to Miss Elizabeth Ingalls for technical assistance in conducting the experiments and for preparing the hemocyanin solutions employed.

590 mμ. This measurement is not corrected for absorption by the solvent. The fourth column records the extinction coefficient of the oxygenated chromatic groups, $2/d(\log \tan a_y - \log \tan a_r)$; the fifth column, the value of $y$ as defined in equation (7). The equilibration was carried out in a water bath at 25° C. The length of the T-tube of the tonometer in which the absorption of light was measured, $d$, was usually 3.3 centimeters. In the case of a few measurements, tubes were employed which differed slightly from this length (3.15 to 3.60 cm.).

## Theory of Oxygen Equilibrium

In oxyhemocyanin, one molecule of oxygen is bound by a quantity of hemocyanin containing two atoms of copper. The reversible reaction may consequently be indicated by the equation

$$RCu_{2n}O_{2n} = RCu_{2n} + nO_2,$$

where $n$ represents the number of mols of oxygen bound by each mol of hemocyanin. In treating the equilibrium according to the mass law, as was done by Hüfner (1901) and later by Hill (1910), in the case of hemoglobin, the result is

$$\frac{(RCu_{2n}O_{2n})}{(RCu_{2n})(O_2)^n} = k, \tag{8}$$

in which $k$ is the equilibrium constant of the reaction. If $y$ is the fraction of hemocyanin in the oxygenated condition, $1 - y$ is the reduced fraction and, putting $p$, the partial pressure of oxygen in mm. of mercury, in place of the oxygen concentration, equation (8) may be written

$$\frac{y}{1-y} = Kp^n \tag{9}$$

or,

$$\log\left(\frac{y}{1-y}\right) = \log K + n \log p. \tag{10}$$

In this form the equation is convenient for graphical solution for $n$ and $K$.

In Fig. 2 is reproduced the data recorded in Table I, arranged in the form indicated by equation (10). The lines drawn through the points in each case are straight lines indicating the linear relationship demanded by the equation. The slope of the lines drawn through the points, determining the value of $n$, is 1.0. The values of $K$ corresponding to the positions of the lines drawn in Fig. 2 are indicated in Table III. Employing these values of $K$ and taking $n$ as equal to 1.0 in each case, the values of $y$ may be calculated and are indicated in column 6 of

Table III for comparison with the observed values. It appears that the theoretical treatment from which equation (10) is derived is adequate to account for the shape of the oxygen dissociation curve at least

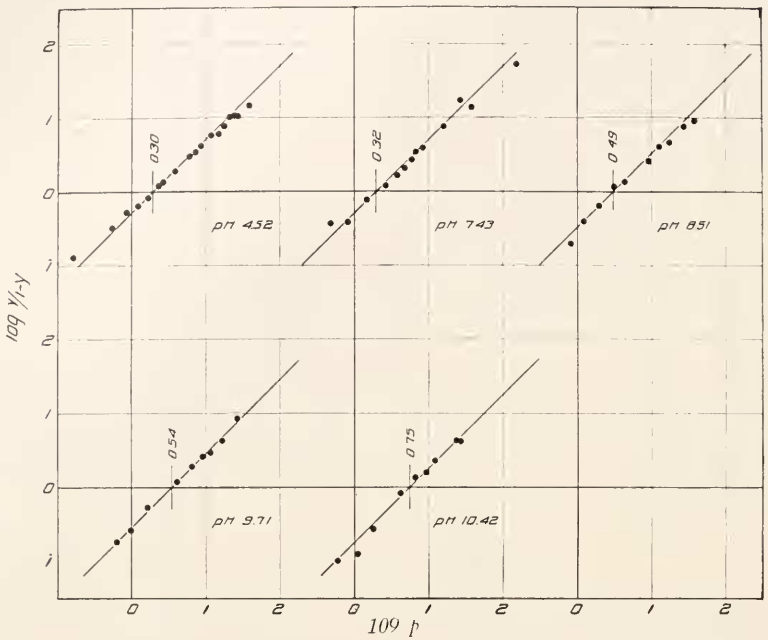


FIG. 2. Logarithmic plot of data of oxygen dissociation curve of hemocyanin of *Limulus polyphemus* at various pH values. Temperature, 25° C.; $y$, fraction of hemocyanin in oxygenated condition; $p$, oxygen pressure in mm. Hg.

as a first approximation, and to provide a single series of constants to define the effect of hydrogen ion activity upon the equilibrium.[3] Careful scrutiny of the data in Table III reveals a tendency for the low values of $y$ to be slightly greater than the calculated values and high values to be slightly less than the theoretical. In order to make vivid the adequacy of the theory for treating the entire set of observations, in Fig. 3 the values of $y$ obtained at each pH value are plotted against $Kp$ in the usual form of the oxygen dissociation curve, and a line corresponding to the theoretical treatment is drawn through the points, $n$ again being taken as 1.0.

[3] It should be emphasized that the pH values are determined on reduced solutions. No account has been taken of possible change in pH with oxygenation. According to Redfield, Humphreys and Ingalls (1929), the effect may be expected to be small.

## Table III

*Data of Oxygen Dissociation Curves of Limulus Hemocyanin.* Temperature, 25° C.; Wave-length, 590 mμ.

| Description | $p$ | $\frac{2}{d}\log\tan a_y$ | $\frac{2}{d}\log\frac{\tan a_y}{\tan a_r}$ | $y$ | $y$ |
|---|---|---|---|---|---|
| | *mm. Hg* | | | *(observed)* | *(calculated)* |
| Specimen 18 *A* | 0 | 0.034 | 0 | 0 | 0 |
| | 0.16 | 0.068 | 0.034 | 0.117 | 0.074 |
| Concentration: | 0.56 | 0.106 | 0.072 | 0.248 | 0.218 |
| 0.0258 grams per | 0.80 | 0.133 | 0.099 | 0.342 | 0.286 |
| cc. | 1.24 | 0.149 | 0.115 | 0.397 | 0.382 |
| Combined acid: | 1.65 | 0.166 | 0.132 | 0.455 | 0.452 |
| $20 \times 10^{-5}$ mols | 2.34 | 0.194 | 0.160 | 0.552 | 0.540 |
| per gram | 2.74 | 0.204 | 0.170 | 0.586 | 0.578 |
| pH 4.52 | 3.92 | 0.225 | 0.191 | 0.659 | 0.662 |
| | 6.20 | 0.252 | 0.218 | 0.752 | 0.757 |
| $K = 0.500$ | 7.28 | 0.259 | 0.225 | 0.776 | 0.784 |
| | 8.65 | 0.269 | 0.235 | 0.810 | 0.812 |
| | 11.9 | 0.281 | 0.247 | 0.852 | 0.856 |
| | 14.8 | 0.283 | 0.249 | 0.859 | 0.881 |
| | 17.6 | 0.291 | 0.257 | 0.886 | 0.898 |
| | 20.6 | 0.298 | 0.264 | 0.910 | 0.913 |
| | 24.5 | 0.299 | 0.265 | 0.914 | 0.924 |
| | 27.4 | 0.299 | 0.265 | 0.914 | 0.933 |
| | 39.0 | 0.305 | 0.271 | 0.935 | 0.952 |
| | 744 | 0.324 | 0.290 | 1.00 | 1.00 |
| Specimen 18 *A* | 0 | 0.034 | 0 | 0 | 0 |
| | 0.39 | 0.092 | 0.058 | (0.265) | 0.171 |
| Concentration: | 0.80 | 0.119 | 0.085 | 0.305 | 0.276 |
| 0.0258 grams per | 1.49 | 0.158 | 0.124 | 0.441 | 0.415 |
| cc. | 2.61 | 0.188 | 0.154 | 0.548 | 0.554 |
| Combined base: | 3.74 | 0.207 | 0.173 | 0.616 | 0.640 |
| $19 \times 10^{-5}$ mols | 4.78 | 0.223 | 0.189 | 0.672 | 0.695 |
| per gram | 5.96 | 0.239 | 0.205 | 0.730 | 0.739 |
| pH 7.43 | 6.83 | 0.253 | 0.219 | 0.780 | 0.766 |
| | 8.38 | 0.258 | 0.224 | 0.797 | 0.800 |
| $K = 0.476$ | 16.6 | 0.282 | 0.248 | 0.882 | 0.888 |
| | 26.4 | 0.300 | 0.266 | 0.946 | 0.927 |
| | 37.7 | 0.297 | 0.263 | 0.936 | 0.948 |
| | 152 | 0.310 | 0.276 | 0.982 | 0.987 |
| | 740 | 0.315 | 0.281 | 1.00 | 1.00 |

TABLE III (*continued*)

*Data of Oxygen Dissociation Curves of Limulus Hemocyanin.* Temperature, 25° C.; Wave-length, 590 mμ.

| Description | $p$ | $\frac{2}{d}\log\tan a_y$ | $\frac{2}{d}\log\frac{\tan a_y}{\tan a_r}$ | $y$ | $y$ |
|---|---|---|---|---|---|
| | *mm. Hg* | | | *(observed)* | *(calculated)* |
| Specimen 18 *B* | 0 | 0.046 | 0 | 0 | 0 |
| | 0.84 | 0.078 | 0.032 | 0.166 | 0.213 |
| Concentration: | 1.22 | 0.103 | 0.057 | 0.295 | 0.282 |
| 0.0208 grams per | 1.98 | 0.123 | 0.077 | 0.399 | 0.390 |
| cc. | 3.15 | 0.150 | 0.104 | 0.539 | 0.504 |
| Combined base: | 4.40 | 0.158 | 0.112 | 0.580 | 0.587 |
| $39 \times 10^{-5}$ mols | 9.30 | 0.185 | 0.139 | 0.720 | 0.750 |
| per gram | 12.80 | 0.201 | 0.155 | 0.803 | 0.805 |
| pH 8.51 | 17.7 | 0.204 | 0.158 | 0.818 | 0.852 |
| | 28.1 | 0.217 | 0.171 | 0.886 | 0.900 |
| $K = 0.322$ | 39.0 | 0.219 | 0.173 | 0.902 | 0.927 |
| | 751 | 0.239 | 0.193 | 1.00 | 1.00 |
| Specimen 18 *C* | 0 | 0.047 | 0 | 0 | 0 |
| | 0.63 | 0.082 | 0.035 | 0.155 | 0.177 |
| Concentration: | 0.97 | 0.093 | 0.046 | 0.204 | 0.217 |
| 0.0242 grams per | 1.64 | 0.128 | 0.081 | 0.358 | 0.319 |
| cc. | 4.12 | 0.167 | 0.120 | 0.531 | 0.541 |
| Combined base: | 6.38 | 0.197 | 0.150 | 0.654 | 0.646 |
| $63 \times 10^{-5}$ mols | 8.94 | 0.209 | 0.162 | 0.717 | 0.719 |
| per gram | 11.5 | 0.216 | 0.169 | 0.748 | 0.767 |
| pH 9.71 | 16.8 | 0.230 | 0.183 | 0.810 | 0.828 |
| | 21.8 | 0.228 | 0.181 | (0.801) | 0.862 |
| $K = 0.286$ | 26.7 | 0.250 | 0.203 | 0.898 | 0.885 |
| | 724 | 0.273 | 0.226 | 1.00 | 1.00 |
| Specimen 18 *C* | 0 | 0.066 | 0 | 0 | 0 |
| | 0.61 | 0.084 | 0.018 | 0.092 | 0.098 |
| Concentration: | 1.10 | 0.088 | 0.022 | 0.112 | 0.164 |
| 0.0242 grams per | 1.79 | 0.124 | 0.058 | 0.296 | 0.242 |
| cc. | 4.15 | 0.155 | 0.089 | 0.454 | 0.426 |
| Combined base: | 6.53 | 0.177 | 0.111 | 0.566 | 0.538 |
| $77 \times 10^{-5}$ mols | 9.04 | 0.183 | 0.117 | 0.597 | 0.618 |
| per gram | 12.2 | 0.202 | 0.136 | 0.694 | 0.686 |
| pH 10.42 | 17.1 | 0.242 | 0.176 | (0.898) | 0.754 |
| | 22.6 | 0.225 | 0.159 | 0.812 | 0.802 |
| $K = 0.178$ | 27.2 | 0.224 | 0.158 | 0.806 | 0.830 |
| | 751 | 0.262 | 0.196 | 1.00 | 1.00 |

## Discussion

In the forty years since Hüfner suggested the application of the mass law to the equilibrium between oxygen and hemoglobin, numerous investigations have indicated that equations similar to those employed in the present treatment are more or less adequate to describe the data in hemoglobin solutions free of electrolytes (Barcroft, 1928). Uncertainty has sometimes accompanied the results of such investigations because of the instability of purified hemoglobin solutions (Ferry, 1924; Hecht, Morgan and Forbes cited by Barcroft, 1928). In the presence of electrolytes and in blood, the dissociation curves of hemoglobin invariably have a sigmoid shape, requiring some additional assumptions for their explanation.

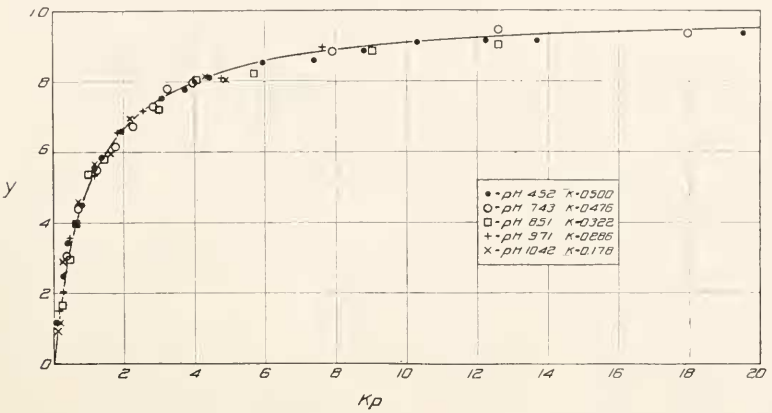


Fig. 3. Data of oxygen dissociation curves of hemocyanin of *Limulus polyphemus* plotted to show the similarity of shape at various pH values. $y$ is fraction of hemocyanin present as oxyhemocyanin; $p$ is oxygen pressure in mm. Hg; temperature, 25° C. The curve corresponds to equation (9) when $K = 1$, $n = 1$.

In the case of hemocyanin solutions, Stedman and Stedman (1928) found that the respiratory pigment of the snail, *Helix pomatia*, combines oxygen in accordance with the mass law, as expressed in equation (8), $n$ being taken to be 1.0. The hemocyanin of the crustacea, *Homarus vulgaris* and *Cancer pagurus*, according to these investigators (1926 *a*, 1926 *b*) is characterized by oxygen dissociation curves of a more complex nature when examined in dialyzed solution. The present investigation of *Limulus* hemocyanin indicates that this substance, when in "salt free" solutions, resembles the hemocyanin of *Helix* in its conformity to the mass law.

Stedman and Stedman, in discussing their observations on *Helix*

hemocyanin, conclude from the fact that the value of $n$ is 1.0, that this hemocyanin is dispersed in solution in such a way that each hemocyanin molecule unites with but a single oxygen molecule. It is tempting to draw the same conclusion with regard to *Limulus* hemocyanin, for the investigations of Redfield, Coolidge and Shotts (1928) indicated that the probable molecular weight of this protein is 73,400 and that each molecule contains two atoms of copper. The measurements of Redfield, Coolidge and Montgomery (1928) demonstrate further that such a hemocyanin molecule would bind but a single oxygen molecule. The value of $n$ established in this investigation follows as a prediction from these considerations. It must be recalled, however, that Svedberg and Heyroth (1929) obtained much larger values for the molecular weight of *Limulus* hemocyanin by the employment of the ultra-centrifugal method. In view of the uncertainty regarding the size of the hemocyanin molecule, reserve is required in interpreting the data of the oxygen equilibrium. If one goes back to the kinetic basis of the mass law equation (8), it may be noted that the fundamental assumptions concern the probability of the union of an oxygen molecule with the respiratory protein and the probability of the dissociation of such a union. Where expressions arise giving values of $n$ greater than 1.0, or more complicated equations, it is through the assumption that some relation exists between the combination of oxygen by contiguous groups; either that they unite with oxygen simultaneously as pairs or larger groups, or that they combine in successive steps so that one cannot react until after others have done so. All that can safely be concluded from a demonstration that hemocyanin unites with oxygen as though it were dispersed in molecules each combining with but a single oxygen molecule is that it behaves *as though* this were the case. That is to say, the oxygen dissociation curve is such as would be obtained if the various oxygen binding groups reacted independently of one another so that the oxygenation of any one did not influence the probability of oxygenation or reduction of any other. That this may be the case in a molecule containing a number of oxygen-binding groups does not seem altogether impossible when it is recalled that the molecular weight of such a molecule would be 73,400 times the number of groups. It should be recalled that in combining with acid, the molecule of *Limulus* hemocyanin, which binds at least 117 equivalents of acid, behaves as though each acid-binding group reacted independently of every other (Redfield and Mason, 1928). In this regard the behavior of this protein is not exceptional.

The measurements recorded in Table III make it clear that as alkali

is added to solutions of purified *Limulus* hemocyanin the equilibrium constant of oxygenation decreases progressively, indicating that greater pressures of oxygen are required to produce any given degree of oxygenation. No suggestive relationship is apparent between the values of $K$, and either the quantity of alkali added or the hydrogen ion activity of the solution. It is noteworthy that the phenomena exhibited by these purified solutions of hemocyanin differ markedly from those obtaining in the native serum of *Limulus*. As pointed out briefly by Hogben and Pinhey (1927) an extensive series of measurements on the oxygen dissociation curves of *Limulus* serum (which we have not published) demonstrate that at pH values up to about 8.3 the oxygen pressure requisite to produce a given degree of oxygenation increases. At higher pH values these pressures decrease again, much as is the case with *Helix aspersa* blood (Hogben and Pinhey, 1926).

In their investigation of the dialyzed hemocyanin of *Helix pomatia*, Stedman and Stedman (1928) report that no detectable change in the curve with change in pH was observed. Experiments now in progress with the purified hemocyanin of *Busycon canaliculatum* agree closely with the findings in the case of *Limulus*, indicating a definite decrease in the value of $K$ with diminishing hydrogen ion activity. Inasmuch as the results with *Helix* are otherwise very similar to those obtained with *Limulus* and *Busycon* hemocyanin, we have reëxamined the Stedmans' data and find evidence suggesting that, with this material, there may be a small effect of hydrogen ion concentration upon the value of $K$. In a set of curves defined by equation (9), differing only in the value of $K$ and where $n$ equals 1.0, the greatest differences in $y$ obtain between degrees of saturation of 0.40 and 0.80. It is in this range that differences in the curves may be most readily detected. We have consequently evaluated $K$ on the basis of their data selected between these degrees of saturation. The results are presented in Table IV. The average value of $K$ for the data selected is 0.250. Of the nine measurements made on solutions more alkaline than pH 7, the mean value is 0.217 and the highest value is 0.236. Of the ten measurements made on solutions more acid than pH 7, the average value is 0.270, and only two values are less than 0.236. This result indicates that a small but definite change in the value of $K$ may occur in the case of *Helix* hemocyanin with change in hydrogen ion concentration, and that the phenomena in this case may not differ qualitatively from that obtaining with *Limulus* and *Busycon*.

TABLE IV

*The Equilibrium Constant of Oxygenation of* HELIX POMATIA *Hemocyanin at Various pH Values Calculated from the Data of Stedman and Stedman (1928)*

| *pH* | *p* | Per cent Saturation (corrected) | Per cent Unsaturated | *K* |
|---|---|---|---|---|
| | *mm. Hg* | $100 \times y$ | $100 \times (1 - y)$ | |
| 4.04 | 2.89 | 47.9 | 52.1 | 0.318 |
| | 8.38 | 70.3 | 29.7 | 0.282 |
| | 11.61 | 72.4 | 27.6 | 0.226 |
| 4.79 | 2.74 | 40.4 | 59.6 | 0.247 |
| | 6.73 | 67.9 | 32.1 | 0.314 |
| 6.25 | 2.85 | 46.7 | 53.3 | 0.307 |
| | 6.45 | 64.5 | 35.5 | 0.282 |
| | 11.13 | 70.8 | 19.2 | 0.331 |
| 6.35 | 3.79 | 47.9 | 52.1 | 0.243 |
| | 11.30 | 62.6 | 37.4 | 0.148 |
| 7.81 | 2.83 | 39.8 | 60.2 | 0.234 |
| | 7.00 | 57.1 | 42.9 | 0.190 |
| | 7.96 | 62.8 | 37.2 | 0.212 |
| 8.74 | 2.90 | 40.6 | 59.4 | 0.236 |
| | 6.15 | 59.1 | 40.9 | 0.235 |
| | 10.04 | 65.8 | 34.2 | 0.191 |
| 9.02 | 2.50 | 37.0 | 63.0 | 0.235 |
| | 6.00 | 55.6 | 44.4 | 0.209 |
| | 7.51 | 62.1 | 37.9 | 0.218 |

## SUMMARY

A spectrophotometric method for measuring the equilibrium of hemocyanin and oxygen is described.

The oxygen dissociation curves of purified hemocyanin of *Limulus* in the absence of salts and at various hydrogen ion activities are determined.

It is shown that the equilibrium between oxygen and these hemocyanin solutions is defined, as a first approximation, by the mass law on the assumption that the various oxygen-combining groups react independently of one another in their combination with oxygen.

The value of the equilibrium constant of the oxygenation reaction decreases as the pH value increases from 4.5 to 10.4.

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